

TABLE 5. RANGE AND AVERAGE VALUES (AREA PERCENT) FOR FAME OF *E. coli* FROM GOOSE SAMPLES.

[illegible]

TABLE 6. RANGE AND AVERAGE VALUES (AREA PERCENT) FOR FAME OF *E. coli* FROM MUSKRAT SAMPLES.

[illegible]

TABLE 7. AVERAGE VALUES FOR ALL SPECIES.

FATTY ACID	HUMAN	MUSKRAT	RACCOON	DEER	OTTER	GOOSE
C12:0	2.75	2.93	2.94	2.97	2.72	2.96
C13:0	0.14	0.07	0.10	0.07	0.08	0.08
3-OH 12:0	0.04	0.03	0.02	0.03	0.03	0.04
C14:1	0.45	0.41	0.43	0.39	0.44	0.41
C14:0	7.24	7.00	6.44	7.23	6.95	6.41
uk1	0.49	0.83	0.54	0.82	0.78	0.79
ai 15:0	0.04	0.01	0.01	0.01	0.01	0.00
C15:0	0.89	0.41	0.51	0.47	0.52	0.45
2-OH 14:0	0.20	0.19	0.22	0.16	0.24	0.20
3-OH 14:0	5.41	6.20	5.59	6.13	6.33	6.44
16:1w7	3.94	3.18	4.05	4.22	4.20	4.44
C16:0	31.43	30.39	31.11	30.34	30.65	30.06
iso 17:0	0.02	0.02	<0.01	0.03	0.02	<0.01
C17:1	0.07	<0.01	0.03	0.04	0.06	0.01
17:0 cyc	20.70	21.52	21.81	20.35	21.39	19.93
C17:0	0.43	0.22	0.28	0.24	0.27	0.27
2-OH 16:0	0.01	0.01	0.03	0.05	0.05	0.04
uk2	0.36	0.47	0.42	0.44	0.42	0.44
18:1w7	7.54	5.59	6.28	7.09	6.97	7.60
C18:0	0.57	0.58	0.53	0.57	0.58	0.58
19:0 cyc	11.55	14.37	13.28	13.43	12.97	12.43
C19:0	0.09	0.21	0.04	0.13	0.16	0.18
C20:0	<0.01	<0.01	<0.01	<0.01	0.01	<0.01

TABLE 8. Comparison of average percentages for *E. coli* fatty acids from 5 species: Simmons (S) analyses vs Charleston (C) analyses.

	HUMAN		GOOSE		DEER		OTTER		RACOON	
	S (n=93)	C (n=6)	S (n=32)	C (n=5)	S (n=39)	C (n=6)	S (n=4)	C (n=6)	S (n=46)	C (n=6)
C10:0	0.15		0.16		0.15		0.09		0.09	
C12:0	4.39	2.75	4.71	2.96	4.56	2.97	4.48	2.72	4.54	2.94
C13:0		0.14		0.08		0.07		0.08		0.1
3-OH 12:0		0.04		0.04		0.03		0.03		0.02
C14:1		0.45		0.41	0.01	0.39		0.44	0.01	0.43
C14:0	11.01	7.24	11.83	6.41	11.46	7.32	11.93	6.95	11.41	6.44
uk		0.49		0.79		0.82		0.78		0.54
ai 15:0		0.04		0.00		0.01		0.01		0.01
C15:0	0.25	0.89	0.05	0.45	0.04	0.47		0.52	0.02	0.51
2-OH 14:0		0.20		0.20		0.16		0.24		0.22
3-OH 14:0		5.41		6.44		6.13		6.33		5.59
c16:1w7	3.17	3.94	2.61	4.44	3.08	4.22	2.71	4.20	2.79	4.05
c16:1w5					0.01					
C16:0	37.05	31.43	36.45	30.06	37.59	30.34	37.19	30.65	37.88	31.11
Iso 17:0		0.02				0.03		0.02		
C17:1		0.07		0.01		0.04		0.06		0.03
16:0 DMA	0.03									
17:0 cyc	14.52	20.70	14.82	19.93	14.64	20.35	17.62	21.39	14.73	21.81
C17:0	0.04	0.43	0.02	0.27	0.03	0.24		0.27	0.01	0.28
2-OH 16:0	0.1	0.01	0.21	0.04	0.23	0.05		0.05	0.19	0.03
c18:2w6	0.01									
c18:1w9	0.24									
uk2		0.39		0.44		0.44		0.42		0.42
18:1w7		7.54		7.60		7.09		6.97		6.28
C18:0	0.9	0.57	0.88	0.58	0.90	0.57	0.40	0.58	0.91	0.53
18:0DMA	0.02									
19:0 cyc	12.15	11.55	13.00	12.43	12.59	13.43	13.42	12.97	12.64	13.28
C19:0		0.09		0.18		0.13		0.16		0.04
C20:0								0.01		
summed feature 5	8.02		8.54		7.76		6.16		7.85	
summed feature 11	7.74		6.35		6.55		6.00		6.62	
total	99.79	94.39	99.63	93.77	99.60	95.29	100.00	95.86	99.69	94.66

Figure 1. Principal component analysis of wildlife and human *E. coli* Fatty Acid Profiling results. These fatty acid results explained some 44% of the total variance within the data. Note the significant spatial separation of wildlife (D=Deer, G=Goose, M=Musk rat, O=Otter, and R=Raccoon) and human (H=Human) fatty acid that Principal Component Analysis provides. More than 96% (25/26) of the wildlife values fell within the wildlife Principal Component, indicating that this method may be useful in discriminating wildlife versus human coliform bacterial sources..

Plot of PRIN2*PRIN1. Symbol is value of SAMPLE.

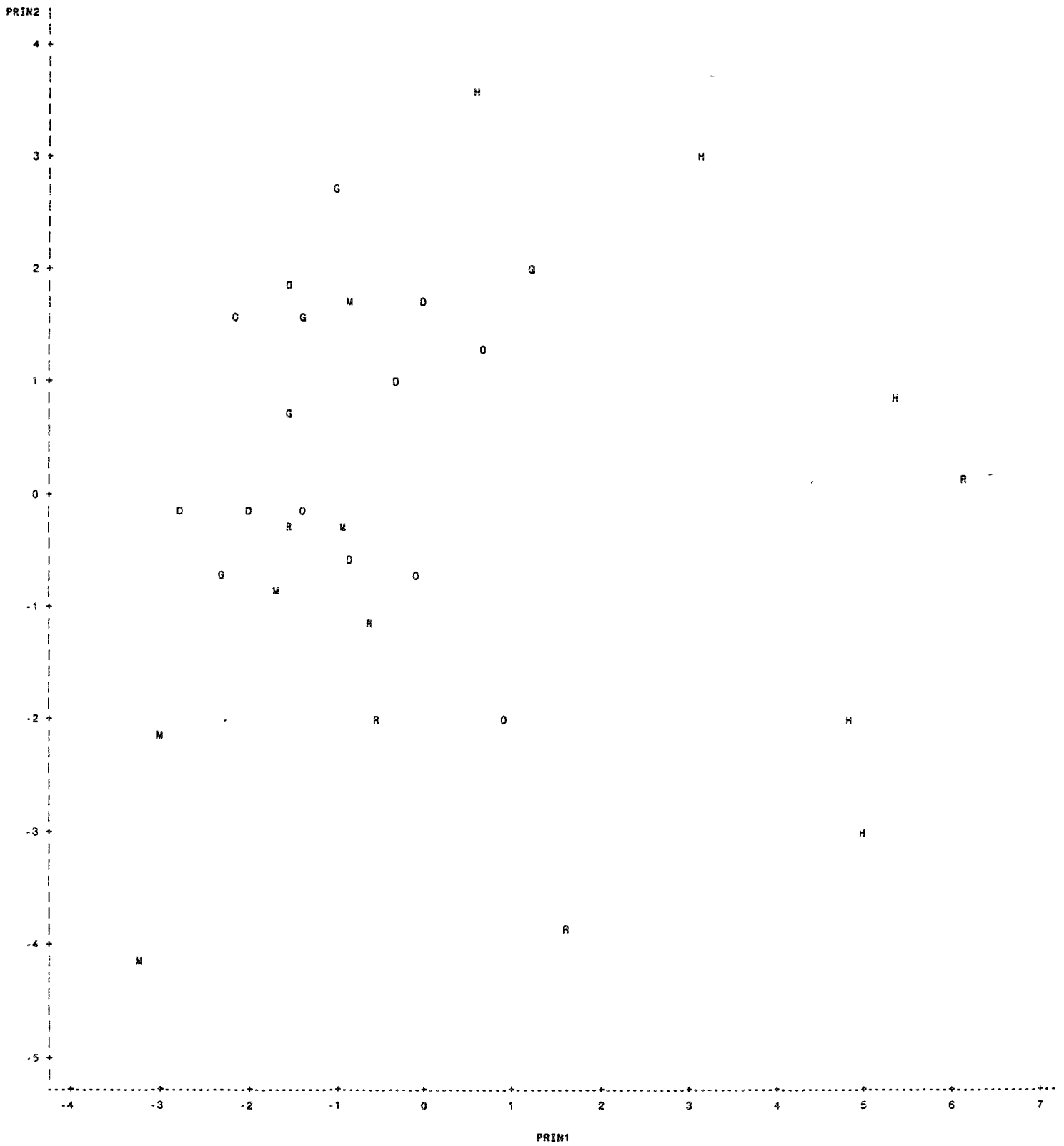


Figure 1

***“PULSED FIELD GEL ELECTROPHORESIS (PFGE)
ANALYSIS OF SELECTED ENVIRONMENTAL E. COLI
SAMPLES TO DISTINGUISH HUMAN VERSUS
WILDLIFE SOURCES”***

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PROGRESS REPORT ON COLIFORM PROJECT

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Fecal coliforms and particularly fecal *E. coli*, have long been used as indicators of fecal pollution of molluscan shellfish and environmental waters. They have become synonymous with the presence of fecal contamination, although the source of contamination is frequently uncertain. Septic systems are often implicated in contamination events, however wild and domestic animals undoubtedly contribute substantially to environmental pollution. Currently, analytical techniques have not been developed to differentiate *E. coli* originating from the feces of animals versus humans. Three approaches were evaluated for the possible identification of *E. coli*'s origin: a) biotyping, where fecal isolates of *E. coli* are subjected to 20 biochemical tests to develop a profile for comparison with other isolates; b) fatty acid profile analyses, to deduce possible differences in the chromatographic profiles of *E. coli* from human versus animal feces; and c) pulsed field gel electrophoresis where the genomic DNA is compared for differences which may be indicative of *E. coli* from humans and animals.

Pulsed Field Gel Electrophoresis: A relatively new technique known as pulsed field gel electrophoresis (PFGE) has been used in epidemiological studies to identify sources of microbial contamination in hospital settings (1,2) and associated with outbreaks of foodborne illness (3-5). PFGE of the *E. coli* isolates involves culturing the microbes, digesting the cultures with proteolytic enzymes to degrade the proteins, and digesting the remaining DNA's with specific restriction enzymes to produce DNA fragments. The fragmented DNA is separated electrophoretically by size on a pulsed field gel and the fragments are visualized on the gel after staining with ethidium bromide. Gels are then photographed and the sizes of the bands are determined by comparison with DNA size standards that are also run on the gel. By visual inspection, it is often possible to identify samples as being the same or different based on these banding profiles. Characterization of profiles can also be performed via the computer when banding profiles become too numerous or complex to visually examine for similarities and differences or when quantitative measurements of band sizes are required.

The concept of applying PFGE to identify the origin of isolates of *E. coli* has been evaluated on dairy farms in Wisconsin (6) and in marine waters along the Chesapeake Bay (7). Working in collaboration with Dr. George Simmons at Virginia Polytechnic Institute and State University, Blacksburg, VA, we evaluated PFGE on *E. coli* isolates obtained from human, deer, raccoon, goose, otter, and muskrat feces provided as stock cultures by Dr. Simmons. Results (Fig. 1) show a banding profile different for each of the species. Our results were statistically compared with the PFGE results obtained for the same isolates in Dr. Simmons' laboratory. The comparison showed that both laboratories provided comparable results. The possibility exists that the Charleston Laboratory may plug directly into the Virginia Polytech database which is rather extensive at this time. This eliminates the need to produce a local database for South Carolina and may expand the local Virginia database to one which is regional.

In a second round of analyses, stools from five volunteers were obtained and five presumptive isolates were cultured from each stool. Biotyping of the isolates was performed using the Analytical Profile Index (API) and confirmed *E. coli* were subjected to PFGE. Figure 2 shows five isolates from one individual (1 A-E) and five isolates from a second person (3 A-E). It is clear from simple visual inspection that all isolates from a particular individual are the same, but that the patterns between individuals are different. Both of these individuals' *E. coli* isolates were of the same biotype. Isolates, representing different biotypes from two other individuals (2 A-E, and 6 B-E), were run on another gel and demonstrated the same patterns in any given person, but different patterns between the people (Fig. 3). A fifth person, whose stools had been cultured, did not display any fecal coliforms. It appears that the biotype does not confer a distinction between human isolates, whereas DNA testing can show great differences. In the process of conducting PFGE, we have optimized parameters for the separation of the DNA fragments on our system by altering running conditions.

On December 4, 1996, members of the Coliform Research Group travelled to Columbia, SC, and met with Dr. George Simmons. We discussed his database and his desire to have us contribute to his database. We will send him *E. coli* isolates from human stools. To date, he reports success in differentiating, with high confidence, *E. coli* from human and animal sources. His comparisons involve isolates from over 60 humans and a variety of different animal species. This exciting news, coupled with our findings that pulsed field gels performed at the Charleston Laboratory are essentially identical to those run at his lab in Virginia, should be viewed with great optimism. PFGE may provide the key to defining important questions on the nonpoint sources of environmental *E. coli* contamination.

Our next set of analyses will focus on *E. coli* isolates obtained from septic tanks and should resolve the question whether human fecal wastes contain many or only a few genetic types of *E. coli*. Although humans possess *E. coli* with many different banding patterns, it is possible that only some *E. coli* can survive the conditions found in septic tanks. If these banding profiles can be determined, it may be possible to identify environmental waters which are impacted by septic waste. Conversely, sites containing *E. coli* with different banding patterns may contain animal feces or fresh human wastes. Further studies to identify *E. coli* from human and specific animals by PFGE are planned for early next year.

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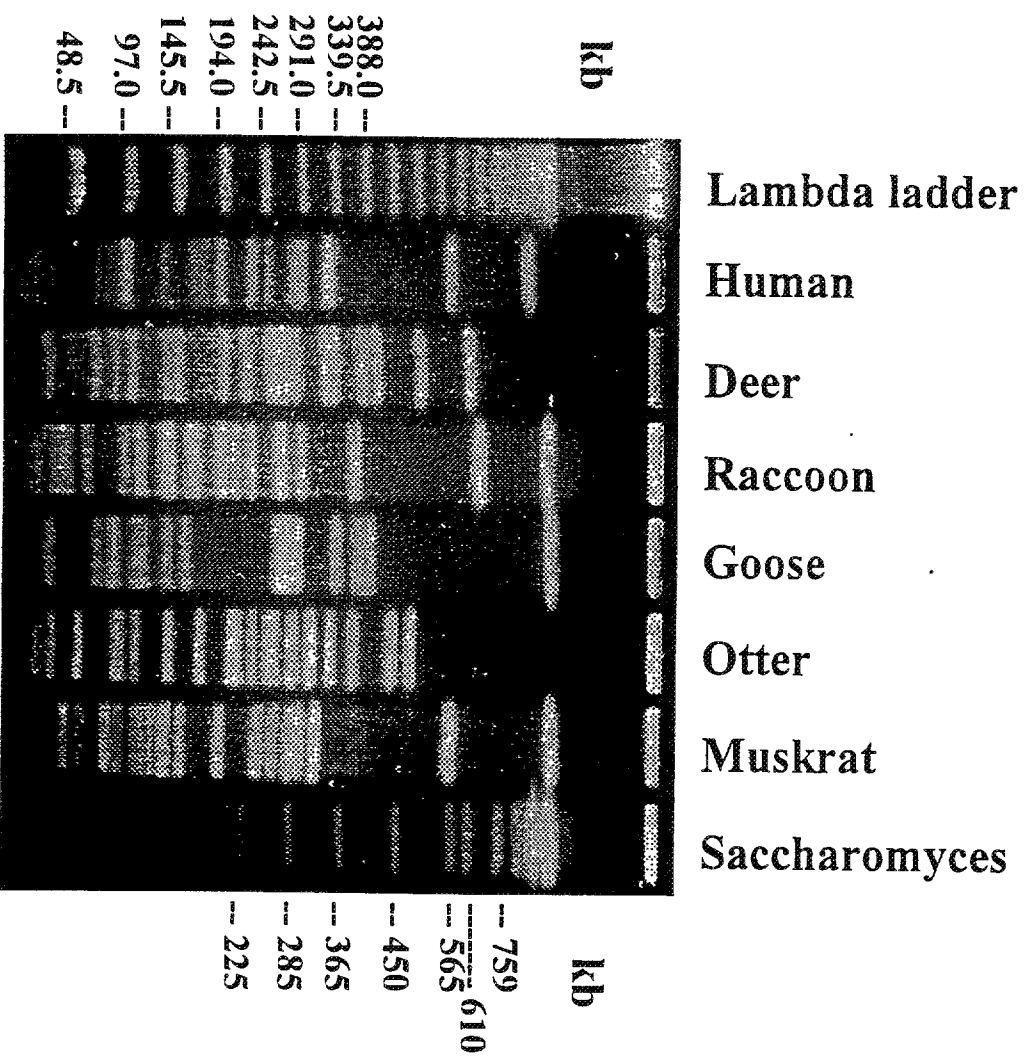


Figure 1. Pulsed Field Gel Electrophoresis of *E. coli* Isolates from Various Animal Sources, *Not I* digests

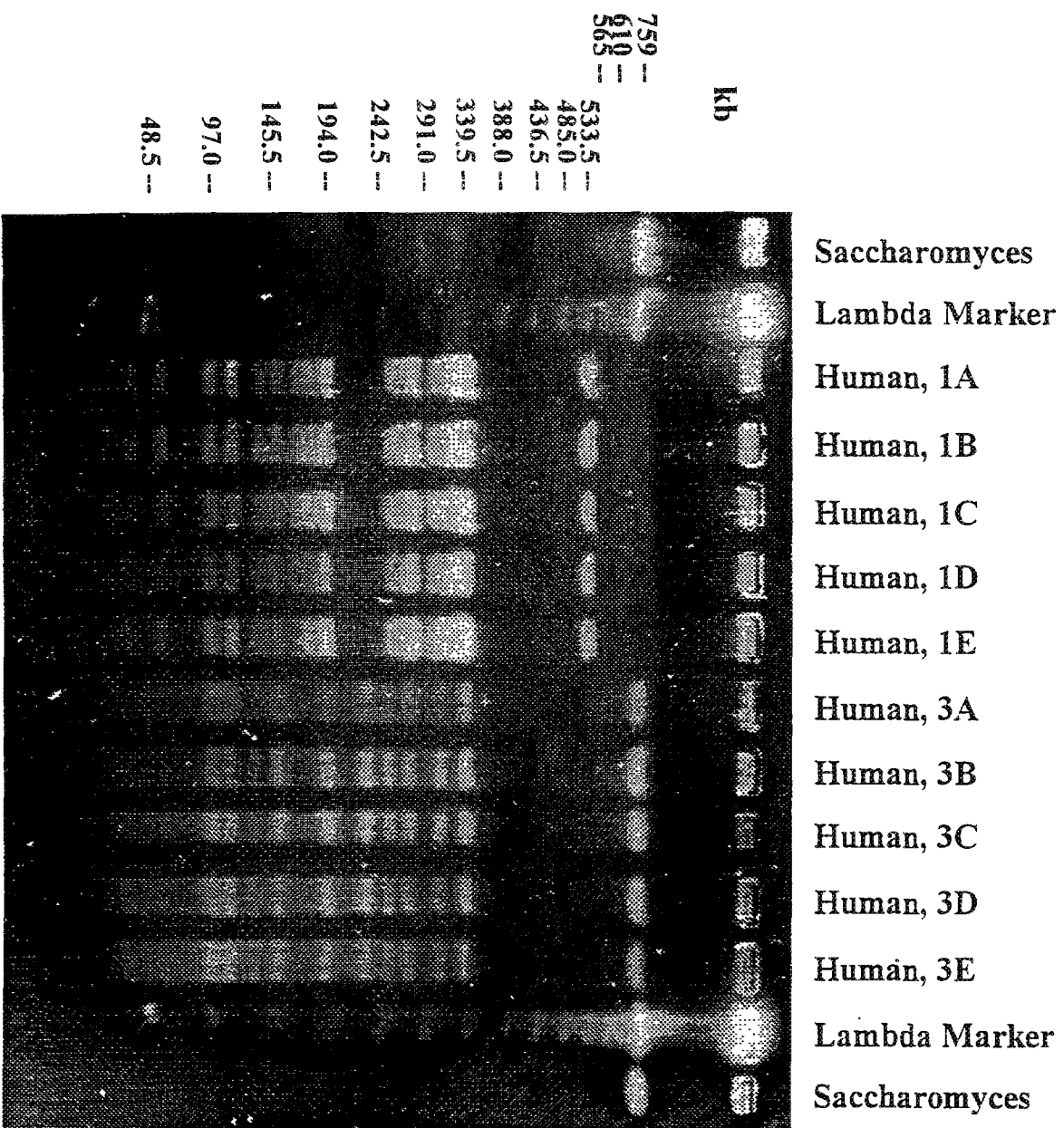
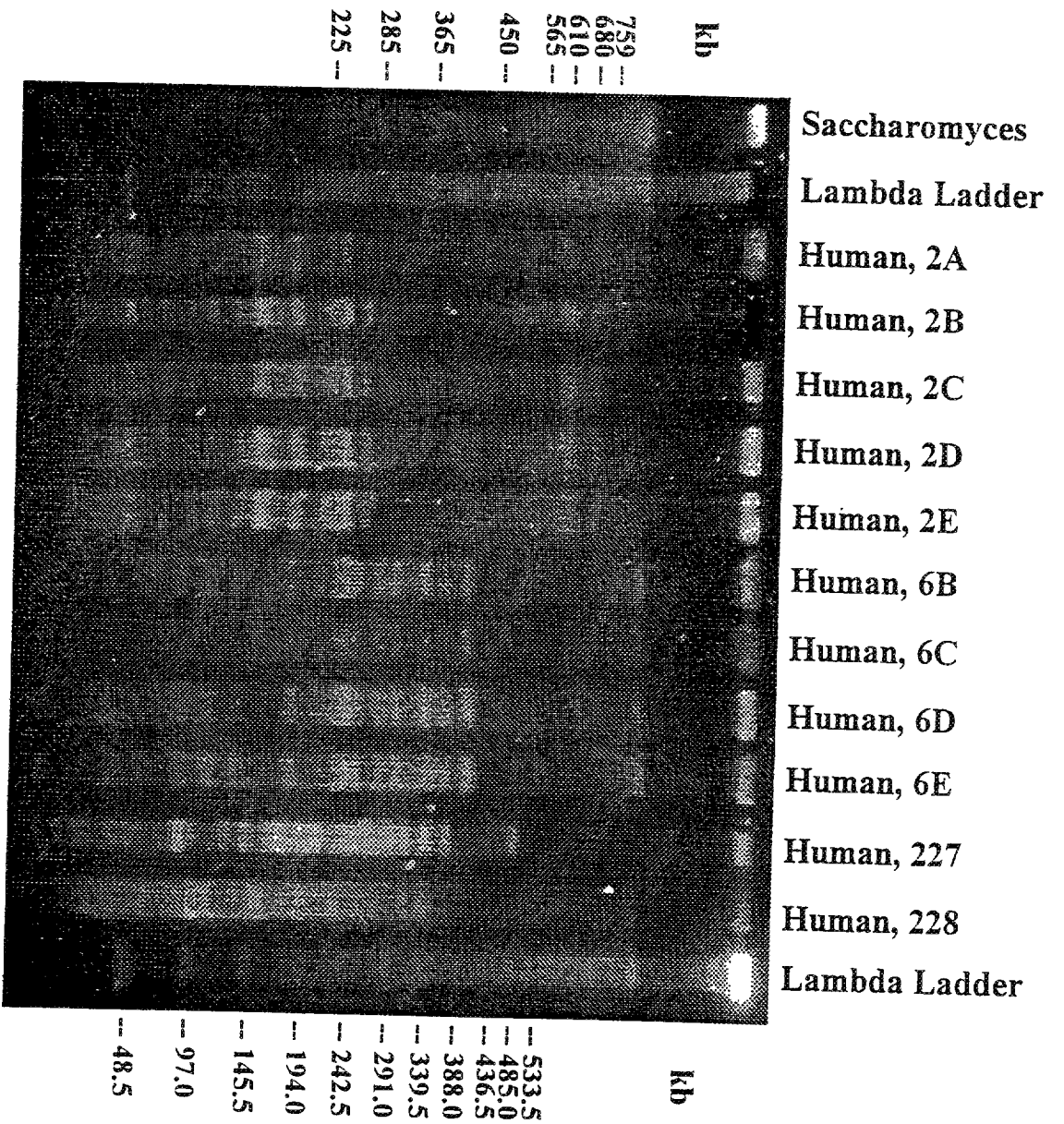


Figure 2. Pulsed Field Gel Electrophoresis of Five *E. coli* Isolates From Each of Two Humans, Not I Digests

**Figure 3. Pulsed Field Gel Electrophoresis
of *E. coli* Isolates, *Not I* Digests**



RESULTS AND DISCUSSION

ANALYTICAL PROFILE INDEX (API) BIOTYPING

Results to date indicated that API profiling was useful in obtaining pure *E. coli* biotype cultures from animals and human samples but that no one API biotype for *E. coli* was specific for human or wildlife samples. Earlier studies conducted by the NMFS measuring API codes in clams from estuaries throughout the state of SC found that three API Codes (5144572, 5144552, and 5044552) accounted for more than 50% of the total API codes at all sites. Additionally, it was found that these three API codes were also associated almost exclusively with urban sampling sites, which suggested that these API codes may be indicative of human *E. coli* biotypes. Our analysis of API codes found that no one code was exclusively found in humans or wildlife samples, rather co-occurred in both human and wildlife samples.

FATTY ACID PROFILING (FAP) ANALYSIS

FAP analysis used extraction methods to saponify and extract fatty acids from *E. coli* samples as fatty acid methyl ethers (FAME). Gas Chromatography-Mass Spectroscopy (GCMS) results identified 21 fatty acids (C12 to C20) in *E. coli* samples which accounted for 95% of the total fatty acid components. Two unidentified fatty acid components were found in all samples which accounted for < 1% of the total fatty acid components. FAP was able to discriminate between human and wildlife samples some 96.2% of the time, using principal component analysis for two selected lipids which accounted for 44% of the total variance in these data. Principal component analysis of FAP was not able to discriminate between different wildlife species, thus we propose to include all wildlife samples as a class when comparing with human isolates. FAP analysis of human *E. coli* isolates grown on broth versus plate media, indicated that culture media greatly influenced FAP results, specifically for the 19:0 cyc lipid fraction (9.78-12.55% in broth versus 4.44-7.07% in plate culture). This indicates that comparison of results from our study with other literature sources involving other bacterial culture methods may be difficult, since culture conditions may greatly affect results.

PULSED FIELD GEL ELECTROPHORESIS (PFGE) ANALYSIS

PFGE results indicated that wildlife and human samples have distinctive bands on gels which may be useful in differentiating between human and wildlife *E. coli* bacterial sources. Comparisons of our data with results from Dr. George Simon's lab at Virginia Tech were quite comparable. Dr. Simon's has determined that when *E. coli* sources could be identified (human versus wildlife= 60% of the time), there was a 90% probability in discriminating wildlife versus human sources. Our results clearly support these findings. PFGE analysis further confirmed that the *E. coli* biotypes found within an individual human fecal swabs sample were unique to that individual. All (100%) male samples contained *E. coli* (biotype codes 7144552 and 7144572). Only 33.3% of the female samples contained *E. coli* (biotype code 1044552) along with

Klebsiella pneumoniae and *Enterobacter sakazakii*. These findings are quite interesting as in the USES Study Scott et al. (1996) reported that the most prevalent API biotypes in urbanized MI were *E. coli* (83%) , *Klebsiella pneumoniae* (6%) and *Enterobacter sakazakii* (3%) accounting for more than 90% of all coliform positive samples.

CONCLUSIONS

API biotyping while useful in isolating different cultures of *E. coli* for PFGE and FAP analysis, is of limited use in differentiating human versus wildlife samples. Conversely, the PFGE and FAP methods have the potential ability to discriminate between human and wildlife sources of *E. coli*. Principal Component statistical analysis of FAP results found that two fatty acid components, which accounted for 44% of the total variance in these data, were useful in discriminating human versus wildlife sources 96.2% of the time in the limited data set of samples analyzed. PFGE analysis has determined that when *E. coli* sources could be identified (human versus wildlife= 60% of the time), there was a 90% probability in discriminating wildlife versus human sources. The combination of PFGE and FAP methodologies appears to be a viable approach for distinguishing human versus wildlife pollution sources. Further validation of PFGE and FAP is on going in septic tank and field samples from watersheds dominated by wildlife pollution sources. Future research should attempt to define the ability of these methods to discern pollution sources in rural areas, where there are low levels of human habitation and abundant wildlife sources, and in urban areas, where there are high levels of human habitation and low levels of wildlife populations. This information would be invaluable to environmental managers to better manage these impacts from urbanization.

FUTURE RESEARCH

The research conducted to date has clearly verified the reproducibility of the PFGE and FAP methods developed by Dr. George Simons at Virginia Tech University. The application of these methods to laboratory stock cultures of wildlife and human *E.coli* biotypes has clearly shown that these methods can distinguish wildlife and human sources. The application of these methods to human fecal swabs from male and female volunteers has demonstrated that there are individual *E. coli* biotypes that are unique to an individual but not to humans *per se*.

Future research should focus on applying these methods to different watershed types including:

1. Rural watersheds with minimal human encroachment and extensive wildlife populations;
2. Suburban watersheds with septic tanks and minimal wildlife populations;
3. Urban watersheds with septic tanks, storm sewers, industrial discharges and minimal wildlife populations;
4. Sewage treatment plant discharges;
5. Domestic animals such as cats and dogs should be evaluated in addition to traditional wildlife sources for *E. coli* biotypes, FAP and PFGE. This is particularly important given the number of residents having pets within the coastal region; and
6. Selective survival of *E. coli* biotypes should be studied in septic tanks to identify if there are selective *E. coli* biotypes that survive better in subsurface ground water and soil conditions. This would provide evidence for septic tank indicator *E. coli* bacteria biotypes to use in future monitoring studies.

Answering these future research questions would greatly improve our understanding of the use of FAP and PFGE methods to discriminate coliform pollution sources.

APPENDIX 1.

Comparison of PFGE results from the NMFS and Virginia Tech University. NMFS results are coded in blue and Virginia Tech results in light brown.. Results are presented on the basis of Identifiable Bands (on a KiloDalton basis) by species (human, deer, raccoon, goose, otter and musk rat) using the C-DNA Library of Dr. George Simons at Virginia Tech University. Note the significant agreement between NMFS and Virginia Tech results .

1. Molecular sizes calculated with SigmaGel -
Gel Scientific Software
2. Column Statistics for S. cerevisiae and E. coli
Gm107 calculated with SigmaPlot from
controls run with our gels.
3. Spread sheet molecular size data comparison
Virginia Tech PFGE ~~_____~~ VT

Charleston PFGE ~~_____~~ Chas

Column Statistics - S. cerevisiae Molecular Size

	-1-	-2-	-3-	-4-	-5-	-6-	-7-
	680 kb	610 kb	565 kb	450 kb	365 kb	285 kb	225 kb
Mean	673.5000	607.7750	566.1500	458.8500	355.5500	281.1000	220.3000
Std.Dev	16.2402	12.9050	11.3557	11.1137	7.5988	5.9649	6.4300
Std.Err	2.5678	2.0405	1.7955	1.7572	1.2015	0.9431	1.0000
95% Conf	5.1940	4.1273	3.6318	3.5544	2.4302	1.9077	2.0000
99% Conf	6.9539	5.5258	4.8624	4.7588	3.2537	2.5541	2.7000
Size	40.0000	40.0000	40.0000	40.0000	40.0000	40.0000	40.0000
Total	2.6900e+4	2.4311e+4	2.2646e+4	1.8154e+4	1.4222e+4	1.1244e+4	8812.0000
Min	645.0000	583.0000	547.0000	435.0000	342.0000	270.0000	203.0000
Max	712.0000	637.0000	588.0000	481.0000	371.0000	297.0000	234.0000
Min.Pos	645.0000	583.0000	547.0000	435.0000	342.0000	270.0000	203.0000
Missing	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Other	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Skewness	0.2892	0.2723	0.1770	0.5202	0.2831	0.5251	0.2000

Human (226) Chas

703.01	733.16
551.69	563.67
332.16	331.70
294.74	287.37
287.26	250.35
245.69	230.13
228.01	194.33
191.35	181.91
173.58	169.49
163.16	137.83
135.98	94.55
103.84	81.80
99.40	70.64
87.76	35.04
76.12	
34.55	
16.26	

Deer (60) Chas

575.62	604.58
475.62	489.16
393.33	415.68
372.15	385.54
328.62	341.97
317.03	325.31
271.83	289.31
255.60	272.76
245.56	252.30
212.85	219.17
188.68	195.06
143.05	145.12
130.88	131.64
101.90	95.08
82.90	81.80
69.10	66.51
23.28	28.67

Raccoon (1) Chas

722.63	775.53
614.24	625.04
369.32	374.91
288.24	298.44
262.48	263.02
226.97	227.20
208.98	208.94
189.09	190.68
157.81	157.80
131.60	130.95
108.35	105.51
94.76	92.42
59.50	61.61
31.06	35.04
8.88	

Goose (100) Chas

770.72	781.37
749.19	405.16
394.75	377.04
376.84	341.97
341.01	284.44
275.95	260.09
258.57	152.69
152.23	135.08
135.58	100.70
112.95	81.80
90.67	68.51
72.32	28.67
38.03	

CHIR (107) Chas

471.81	453.50
439.35	423.04
372.79	361.22
338.00	328.51
300.33	290.90
280.55	268.63
248.40	233.28
225.74	220.67
204.56	195.97
170.95	157.63
137.83	128.73
103.45	99.87
84.45	82.48
55.23	54.71
32.45	31.66
22.29	20.66

Muskrat (59) Chas

719.25	793.06
546.26	562.21
300.98	315.52
266.24	276.65
243.97	252.30
224.54	234.51
175.24	192.14
136.90	149.04
123.83	131.64
110.22	120.64
96.62	106.20
81.95	86.70
73.05	77.54
51.57	57.38
38.47	46.73
27.99	33.45
14.89	24.42

Column Statistics - JM107 Molecular Size

	-1- Band 1	-2- Band 2	-3- Band 3	-4- Band 4	-5- Band 5	-6- Band 6	
Mean	726.2857	504.1429	358.0000	272.0714	244.5000	207.9286	VT
Std.Dev	28.5696	11.6939	8.0288	6.2814	5.8277	5.1510	
Std.Err	7.6355	3.1253	2.1458	1.6788	1.5575	1.3767	
95% Conf	16.4959	6.7520	4.6358	3.6269	3.3649	2.9742	
99% Conf	23.0023	9.4151	6.4642	5.0574	4.6920	4.1473	
Size	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	
Total	1.0168e+4	7058.0000	5012.0000	3809.0000	3423.0000	2911.0000	
Min	695.0000	485.0000	347.0000	264.0000	238.0000	200.0000	
Max	783.0000	530.0000	376.0000	285.0000	258.0000	219.0000	
Min.Pos	695.0000	485.0000	347.0000	264.0000	238.0000	200.0000	
Missing	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
Other	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
Skewness	0.5940	0.6318	0.8607	0.3823	0.9692	0.4000	

Chas

775.53
515.46
361.10
278.60
242.55
208.94
194.33
154.88
134.39
100.01
79.14
32.92

	Band 7	Band 8	Band 9	Band 10	Band 11	Band 12	
Mean	190.0000	152.2143	134.1429	104.0714	84.2143	30.7857	VT
Std.Dev	5.4772	5.6046	4.4351	3.6261	2.6654	5.6457	
Std.Err	1.4639	1.4979	1.1853	0.9691	0.7124	1.5089	
95% Conf	3.1625	3.2361	2.5608	2.0937	1.5390	3.2598	
99% Conf	4.4099	4.5125	3.5709	2.9195	2.1460	4.5455	
Size	14.0000	14.0000	14.0000	14.0000	14.0000	14.0000	
Total	2660.0000	2131.0000	1878.0000	1457.0000	1179.0000	431.0000	
Min	181.0000	143.0000	128.0000	97.0000	81.0000	20.0000	
Max	202.0000	163.0000	143.0000	110.0000	88.0000	41.0000	
Min.Pos	181.0000	143.0000	128.0000	97.0000	81.0000	20.0000	
Missing	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
Other	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
Skewness	0.3673	0.3094	0.4944	-0.4715	0.2171	0.2766	